PROMOTION DE LA CROISSANCE DES PLANTES EN UTILISANT DES SOUCHES DE *STREPTOMYCES* ET DE *BACILLUS* PRODUISANT DE L'AUXINE, SEULES OU EN CONSORTIUM

par

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SOMMAIRE

Le monde est confronté à un problème de sécurité alimentaire dû à la surpopulation mondiale qui conduit à une utilisation accrue des engrais chimiques pour augmenter la productivité agricole. Bien que les engrais chimiques présentent des avantages, ils ont des effets négatifs sur l'environnement et la santé humaine. Cela a suscité un intérêt pour l'utilisation des rhizobactéries qui favorisent la croissance des plantes (RFCP) en tant que principes actifs des biofertilisants. Les RFCP favorisent la croissance des plantes par des mécanismes directs et indirects tels que la production de phytohormones, la fixation de l'azote, la solubilisation du phosphate, la production d'antibiotiques et des enzymes lytiques et la production de sidérophores. Dans la présente étude, nous nous sommes concentrés sur la promotion de la croissance des plantes avec l'acide indole acétique (IAA) produit par RFCP. Le but de cette étude était d'établir pour la première fois des consortiums RFCP formés avec des souches d'actinobactéries et de *Bacillus* produisant de l'IAA. Il a été démontré que les consortiums RFCP offrent une plus grande cohérence que les souches individuelles de RFCP, qui présentent parfois des résultats incohérents dans des conditions réelles. Une technique colorimétrique a été utilisée pour cribler une collection d'isolats d'actinobactéries et de *Bacillus* en vue de la production d'IAA en utilisant le réactif de Salkowski. La capacité des souches les plus productives en IAA à favoriser la croissance de la plante modèle *Lemna minor* a été testée. Respectivement, 73% et 11% des souches d'actinobactéries et de *Bacillus* sélectionnées produisant de l'IAA ont favorisé la croissance de *L. minor*. La technique de superposition de double gélose a été utilisée pour tester la compatibilité entre les souches actinobactériennes et *Bacillus* sélectionnées. Il n'a pas été possible de former des consortiums contenant plus de trois souches en raison de l'antagonisme entre les souches ainsi la plupart des consortiums sélectionnés étaient composés de deux souches. Quatorze consortiums ont été testés et sept d'entre eux ont favorisé la croissance de *L. minor*. La capacité

d'une combinaison d'isolats compatibles à promouvoir le nombre de frondes de *L. minor* était égale ou inférieure à la capacité des souches constituant le consortium. Les consortiums A et E ont également favorisé la croissance des plantules de laitue, indiquant que *L. minor* est une bonne plante modèle pour le criblage de RFCP. La capacité du consortium A à promouvoir la croissance des plantules de laitue était égale à celle de la souche JW 239 seule, tandis qu'une synergie était observée entre les membres du consortium E, ce qui suscitait de l'intérêt pour l'application sur le terrain.

Mots clés : Consortia, *Bacillus*, *Streptomyces,* acide indole-3-acétique, *Lemna minor*, laitue.

SUMMARY

The world is facing a food security problem because of global overpopulation that is leading to increased use of chemical fertilizers to drive agricultural productivity. Although, chemical fertilizers are beneficial, they can have negative impacts on the environment and human health. This has inspired interest in using plant growth promoting rhizobacteria (PGPR) as active ingredients of biofertilizers. PGPR promote plant growth by direct and indirect mechanisms such as phytohormones production, nitrogen fixation, phosphate solubilization, antibiotics and lytic enzyme production and siderophore production. In the present study we focused on promoting plant growth by indole acetic acid (IAA) produced by PGPR. The aim of this study was to establish for the first time PGPR consortia formed with IAA producing actinobacterial and *Bacillus* strains. PGPR consortia have been shown to provide more consistency than individual PGPR strains which sometimes show inconsistent results under field conditions. A colorimetric technique was used to screen a collection of actinobacterial and *Bacillus* isolates for IAA production by using the Salkowski reagent. The ability of the highest IAA producing strains to promote the growth of the model plant *Lemna minor* was tested. Respectively, 73% and 11% of the selected IAA producing actinobacterial and *Bacillus* strains promoted *L. minor* growth. The double agar overlay technique was used to test the compatibility between the selected actinobacterial and *Bacillus* strains. It was not possible to form consortia containing more than three strains due to the antagonism between the strains, therefore most of the selected consortia were composed of two strains. Fourteen consortia were tested and seven of them promoted *L. minor* growth. The capacity of a combination of compatible isolates to promote *L. minor* frond numbers was found to be equal or lower than the capacity of the individual strains composing the consortium. Consortia A and E also promoted lettuce seedlings growth, indicating that *L. minor* is a good model plant to screen PGPR. Ability of consortium A to promote lettuce seedling growth was equal to that of the single strain JW 239 while a synergy

was observed between members of consortium E which suggest that these strains could be of interest for field applications.

Key words: Consortia*, Bacillus, Streptomyces,* indole-3-acetic acid, *Lemna minor*, lettuce.

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LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylate
AHL	Acyl-homoserine lactones
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
B. thuringiensis	*Bacillus thuringiensis*
C	Cytosine
CFU	Colony Forming Unit
DNA	Desoxyribonucleic Acid
dNTP	Deoxyribonucleotide
Fe^{3+}	Trivalent iron
Fe^{2+}	Divalent iron
$FeSO_4 7H_2O$	Ferrous sulfate heptahydrate
G	Guanine
g	Gramme
h	Hour
IAA	Acide indole-3-acétique
IAA	Indole-3-acetic acid
ISR	Induced systemic response
KH_2PO_4	Monopotassium phosphate
l	Litre
L. minor	*Lemna minor*
LSD	Least significant Differance
$MgSO_4$ 7H2O	Magnesium sulfate heptahydrate
min.	Minutes
ml	Milllilitre
Mg	Milligrammes

mM	Millimolar
N_2	Nitrogen fixation
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
$(NH_4)_2SO_4$	Sulfuric acid diammonium salt
P	Phosphorous
P. polymyxa	*Pseudomonas polymyxa*
PCR	Polymerase chain reaction
PGP	Plant growth promoting
PGPR	Plant growth promoting rhizobacteria
pH	Potential hydrogen
rpm	Rotation per minute
RFCP	Des rhizobactéries qui favorisent la croissance
s	Second
S. badius	*Streptomyces badius*
S. D.	Standard deviation
VOCs	Volatile organic compounds
U	Unit
YME	Yeast malt extract
µl	Microlitre

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CHAPTER 1

GENERAL INTRODUCTION

1. The food security problem

Regardless of the increase in agricultural productivity during the last century, today the world faces a food security problem as the number of undernourished people is unacceptably high and the demand for food is continually increasing. This is due to the global over population which is expected to increase by about 35% by 2050 (Obaisi, 2017).

During the last few decades, agricultural production has increased as a result of enhancing consumption of chemical fertilizers which are substances industrially manipulated, composed of known quantities of potassium, phosphorus and nitrogen which is used to add nutrients to the soil to promote soil fertility (Dong *et al*., 2012), increase plant growth and control the damage caused by phytopathogens.

Despite the advantages of chemicals fertilizers, they have also many disadvantages as the frequent exposure of the soil to chemical fertilizers can harden the soil, decrease fertility, pollute air and water and increase the irrigation demand thereby bringing dangers to both human health and environment (Savci, 2012).

The objective of agriculture in the coming period is to modify soil productivity while keeping its ability to function as a healthy system. This has inspired interest in using plant growth promoting rhizobacteria (PGPR) as biofertilizers as the use of the bacteria as efficient inoculants is a safe alternative to chemical fertilizers for improving soil quality without polluting environment and ensuring sustainable crop production at low cost (Mahanty *et al*., 2016).

2. Rhizosphere

The rhizosphere is the narrow zone of soil directly surrounding the root system and it is a favorable habitat for the proliferation of microorganisms as it is influenced chemically, physically and biologically by the plant root (Prashar *et al*., 2013). An important group of these microorganisms that compete for colonizing the root environment are the PGPR which were first defined by Joseph W. Kloepper in the late 1970s. They exert beneficial effects on plant growth and crop yield in several plant species (Adesemoye and Egamberdieva, 2013).

Root exudates are chemical compounds synthesized and secreted by plant roots, and that accumulate in soil. Root exudates, which are rich in monosaccharides, amino acids and organic acids, act as the principal source of nutrients to support the growth and the activities of different microorganisms in the vicinity of the roots. The composition of these exudates is dependent upon the physiological status and species of plants and microorganisms (Doornbos *et al*., 2012). The quality and quantity of root exudates depend on the microbial activity in the rhizosphere which has a great effect on supplying the plants with nutrients (Badri and Vivanco, 2009). Moreover, these exudates promote the plant-beneficial symbiotic interactions and inhibit the growth of competing plant

species (Ahemad and Kibret, 2014). Root exudates act as chemical attractants for a variety of soil microbial communities. In contrast, some of these exudates act as repellants against phytopathogens (Olanrewaju *et al*., 2019).

Rhizosphere colonization is the microbial colonization of the adjacent soil under the influence of the roots (Milus and Rothrock, 1993), while root colonization is the microbial colonization of the rhizoplane and/or root tissues. These root-colonizing microorganisms can be parasitic or saprophytic and free-living, and their diversity is changing, with frequent shifts in community structure and species abundance (Parke, 1991).

3. Plant growth promoting rhizobacteria (PGPR)

PGPR have gained attention as an important group of agriculturally beneficial bacteria of a great commercial interest (Adesemoye and Egamberdieva, 2013). PGPR are free-living rhizosphere bacteria which can colonize plant roots (Allard-Massicotte *et al*., 2016). PGPR can enhance the availability of plant nutrients and decrease the use of chemical fertilization.

PGPRs can be found in several bacterial species such as *Pseudomonas*, *Bacillus*, *Azospirillum*, *Azotobacter*, *Streptomyces*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Flavobacterium*, *Burkholderia*, *Bradyrhizobium*, *Mesorhizobium*, *Rhodococcus* and *Serratia*, which enhance plant growth and yield production (Verma *et al*., 2019). However, the most widely studied bacterial species as PGPR candidates for improvement of plant growth and health are *Pseudomonas* and *Bacillus* (Adesemoye *et al*., 2008). PGPR are classified as extracellular PGPR found in the

spaces between the cells of the root cortex, on the rhizoplane or in the rhizosphere and as intracellular PGPR which found inside root cells and in nodular structures (Gray and Smith, 2005).They can improve plant growth and increase crop yields by different mechanisms (Figueiredo *et al*., 2016). Some examples of these mechanisms, which can be efficient at different stages of plant growth, are: improving the iron uptake by producing siderophores that chelate iron (Gupta and Gopal, 2008), fixing atmospheric nitrogen that is transferred to the plant (Ryu *et al*., 2005), contributing to mineral phosphorous solubilization in soil, improving the crop production (Turan *et al*., 2012). PGPR are known to promote plant growth and health by enhancing their tolerance to a variety of environmental stresses through ACC deaminase production and phytohormone production (Patel and Saraf, 2017). PGPR are reported to compete with pathogens for nutrients (Beneduzi, *et al*., 2012), occupy different niches on the root and improve plant tolerance to drought, salinity (Hussein and Joo, 2018) and metal toxicity.

Usage of PGPR for sustainable agriculture has increased worldwide. It was reported that inoculation with PGPR has increased growth and crop yield of several agronomic crops including tomato (Almaghrabi *et al*., 2013), rice (Sen and Chandrasekhar, 2014), onion (Colo *et al*., 2014) and potato (Otroshy *et al*., 2013). Capacity of PGPR to promote plant growth may be specific to certain plant species cultivars, and genotypes (Lucy *et al*., 2004).

4. Mechanisms of plant growth promotion by PGPR

There is a collection of mechanisms by which PGPR stimulate plant growth. They are classified as direct and indirect mechanisms, as plant growth promoters and biological control agents (Kang *et al*., 2010).

4.1 Direct Mechanisms

PGPRs can directly influence plant growth via nitrogen fixation, phosphate solubilization, phytohormone production and increasing iron availability. The ways by which the PGPR use to influence the plant growth vary from species to species as well as strain to strain.

Organic substances that enhance plant growth are known as plant growth regulators. They promote plant growth by influencing the morphological and physiological processes at very low concentrations (Arshad and Frankenberger,1997). Several microorganisms can produce phytohormones such as auxins, gibberellins, cytokinins, ethylene or abscisic acid. Of note several rhizobacterial genera produce auxins e.g. *Azospirillum*, *Agrobacterium*, *Pseudomonas*, *Bacillus* and *Streptomyces* (Costacurta and Vanderleyden, 1995).

4.1.1 Phytohormone production

Phytohormones are chemical messengers that play a major role in the promotion of plant growth and development. Phytohormones are present in low concentrations, otherwise they would limit plant growth and development or become lethal when uncontrolled (Lymperopoulos *et al*., 2018). Phytohormones shape the plant, affect seed growth and germination, flowering, leaf formation and reduction of senescence of leaves and fruits. Phytohormones also regulate many physiological processes in the plant including cellular division and growth, vegetative and reproductive development and stress responses. Plants adjust the levels of their endogenous phytohormones to decrease the impact of stress caused by growth limiting environmental conditions (de

Garcia Salamone *et al*., 2005). Many PGPR can alter phytohormone levels and thereby influence the plant's hormonal balance and its response to stress (Egamberdieva *et al*., 2017). Phytohormones play an important role in modulating the uptake of nutrients and in mediating response to stress and to pathogens therefore improve crop yield and quality. There are different chemical groups of the basic phytohormones, namely: cytokinins, auxins, gibberellins, abscisic acid, ethylene, polyamines, jasmonates and salicyclic acid (Khan *et al*., 2016).

4.1.2 Nitrogen fixation (N_2)

Nitrogen is important nutrient for plant growth and productivity. N_2 is unavailable to the growing plants although there is about 78% N_2 in the atmosphere. Biological N_2 fixation is the conversion of the atmospheric N_2 into plant-utilizable forms by changing nitrogen to ammonia by nitrogen fixing microorganisms that possess a complex enzyme system known as nitrogenase (Kim and C. Rees, 1994). Some, biological nitrogen fixation occurs at mild temperatures by nitrogen fixing microorganisms that are widely distributed in nature (Raymond *et al*., 2004). Generally, N_2 fixing organisms are symbiotic endophytic that include members of the family rhizobiaceae which form symbiosis with leguminous plants (e.g. rhizobia). This nitrogen fixing rhizobacteria establish symbiosis in the roots of plants through a complex interaction between the host and symbiont resulting in the formation of the nodules (Zahran, 1999). Actinobacteria (*Frankia* sp.) establish a similar (root nodule) symbiosis with non-leguminous, woody plant species (Santi *et al*., 2013). PGPR able to fix N_2 are also called diazotrophs and some can form non-obligate cooperation with host plants (Santi *et al*., 2013). other non-symbiotic N_2 fixing bacteria (e.g. cyanobacteria, *Azotobacter*, *Azospirillum* and *Azocarus*) provide a small amount of fixed nitrogen that bacteria associated with the host plant require (Kavimandan *et al*., 1978). PGPR which can fix N_2 are economically beneficial and environmentally sound alternatives to chemical

fertilizers. Nitrogen-fixing bacteria with multiple plant growth-promoting activities enhance the growth of tomato and red pepper (Islam *et al*., 2013).

4.1.3 Phosphate solubilization

After nitrogen, phosphorous is the most important nutrient for plants. Despite the abundance of soil phosphorus reserves, it is often present in a form unsuitable for plant uptake. Plants are only able to absorb monobasic and dibasic phosphates which are the soluble forms of phosphate. PGPR can mineralize organic phosphorus in soil by solubilizing complex-structured phosphates such as tricalcium phosphate, aluminum phosphate, rock phosphate, etc., which turns organic phosphorous to inorganic form that increase the phosphate availability to plants (Ahemad and Kibret, 2014). These phosphate-solubilizing bacteria use several mechanism(s) to solubilize insoluble phosphate. The major mechanism of phosphate solubilization is based on organic acid secretion by the PGPR through sugar metabolism. PGPR utilize sugars from root exudates and produce organic acids. These acids are excellent chelators of divalent Ca^{2+} cations, thereby releasing phosphates from insoluble phosphate compounds. Many phosphate-solubilizing bacteria lower the pH of the medium by secreting of organic acids such as acetic, lactic, malic, tartaric, gluconic, oxalic and citric acids (Alori *et al*., 2017). The involvement of PGPR in the solubilization of inorganic phosphates has long been known. It is estimated that phosphate-solubilizing bacteria represent1-50% of the proportion of soil and rhizosphere micro-organisms (Sharma *et al*., 2013). The high proportion of phosphate solubilizing bacteria is concentrated in the rhizospheres and is known to be more metabolically active than those isolated from sources other than the rhizosphere (Alori *et al*., 2017). It was reported that *Bacillus megaterium*, *B. sircalmous, B. coagulans*, *B. circulans, B. subtilis*, *Pseudomonas striata* and *P. polymyxa* are some of the most effective phosphate solubilizers (Goswami *et al*., 2013).

Previous studies reported that the co-inoculation of phosphate-solubilizing PGPR strains increased P uptake in chickpea crop compared to control (Gull *et al*., 2004). It was also reported that phosphate solubilizing bacteria isolated from rhizosphere induced colonization and maize growth promotion (Li *et al*., 2017).

4.2 Indirect mechanisms

4.2.1 Antibiotic production and lytic enzymes

PGPR can also use indirect mechanisms to reduce the deleterious effects of phytopathogens on plant growth. They can synthesize the lytic enzymes such as cellulases, chitinases, proteases and lipases that can lyse a portion of the cell walls of several pathogens (Glick, 2012). Also, some antibiotics are produced by PGPR can protect the plants against the proliferation of plant pathogens. The production of one or more antibiotics by PGPR is known to act as antagonistic agents against plant pathogens. PGPR alleviation of pathogenesis by fungal, bacterial and viral agents is documented (Glick, 2012).

4.2.2 Induced systemic response (ISR)

Induced systemic resistance (ISR). is the mechanism of increased resistance at specific sites of plants at which induction had occurred. The defense mechanism of ISR is stimulated as a response to an attack of a pathogen. ISR is not specific to particular pathogen but do protect plants against diseases. PGPR can trigger ISR in plants,

activating pathogenesis-related genes mediated by phytohormone signaling pathways and defense regulatory proteins to prime plants against pathogen attacks (Pieterse et al., 2014). It has been shown that bacterial signaling compounds and microbe-associated molecular triggers such as chitin oligomers, modulate ISR in plants. Pathogen cell-surface factors such as flagellins and O-antigen of lipopolysaccharides elicit ISR, whereas analogs of salicylic acid and jasmonic acid trigger ethylene to elicit NPR1-mediated systemic acquired resistance in plants (Ping and Boland, 2004). For example, acyl-homoserine lactones (AHL)-producing *Serratia liquefaciens* MG1 and *P. putida* IsoF elicited ISR in tomato against *Alternaria alternate* whereas AHL-null mutant strains of both PGPR resulted in reduced ISR (Schuhegger et al., 2006).

4.2.3 Siderophores

Some bacterial strains act as biocontrol agents by producing siderophores. In this case, siderophores produced from PGPR can prevent some plant pathogens from acquiring a sufficient amount of iron thereby limiting their capacity to proliferate (Compant *et al*., 2005). It was reported that this mechanism is effective because biocontrol PGPR are characterized by siderophore production that have much greater affinity for iron than do fungal pathogens (Schippers and Bakker, 1987). Hence, the fungal pathogens were unable to proliferate in the rhizosphere of the host plant due to the lack of iron. Previous studies proved that PGPR siderophores are involved in the suppression of diseases caused by fungal pathogens. It was reported that using mutants which were defective in siderophore production was less effective than using the wild-type strains when attempting to protecting the plants against fungal pathogens (Buysens *et al*., 1996; Martinetti and Loper, 1992). Generally the growth of plants is not affected by the depletion of iron in the rhizosphere caused by PGPR siderophores because most plants can grow at lower iron concentrations than most microorganisms (O'Sullivan and

O'Gara, 1992). In addition, many plants can bind, take up and then utilize iron-siderophore complexes produced by the PGPR (Bar-Ness *et al*., 1991; Y. Wang *et al*., 1993).

5. Signal exchange between plant Roots and PGPR

5.1 Phytohormones produced by PGPR

Phytohormones play a major role in regulating plant growth and development. They are molecular signals that respond to the different environmental conditions. Many rhizosphere bacteria produce hormones for root uptake or manipulate hormone balance in plants to promote growth and stress response. Auxin is a plant hormone and IAA is known to be the most common auxin produced by PGPR. It is involved in the interactions between plants and microbes. At optimal IAA concentrations in plants, application of bacterial IAA may have neutral, positive or negative effects on plant growth (Spaepen and Vanderleyden, 2011). The effect of exogenous IAA is dependent on the levels of endogenous IAA in plants.

It was reported that PGPR which produce IAA induce transcriptional changes in hormone, defense-related and cell wall related genes(Spaepen *et al.,* 2014), increase root biomass, and activate auxin response genes that promote plant growth (Ruzzi and Aroca, 2015).

Some strains of PGPR can produce high amounts of gibberellins and cytokinins which enhance root exudate production and promote plant growth e.g. N_2 fixation bacteria (*Rhizobia, Azorhizobium, Bradyrhizobium, Diazotrpophs)* and phosphate solubilizing bacteria (*Pseudomonas* fluorescens, *Bacillus megatherium* , *Acrobacter acrogens*, *nitrobacter spp*., *Escherichia freundii*, *Serratia* spp., *Pseudomonas striata*, *Bacillus polymyxa)*. Interactions of these hormones with auxins can even alter root architecture (Maheshwari *et al*., 2015).

Ethylene is active at extremely low concentrations in plant tissues: approximately 0.01 to 1.0 part per million (ppm). Lower or higher concentrations have been observed depending on species. For example, some climacteric fruits, such as tomatoes and apples, can generate tens of ppm of ethylene. The accumulation of ethylene in response to stress may increase plant tolerance or stimulate stress-response symptoms and senescence (Iqbal *et al.,* 2017). It was reported that PGPR can promote plant growth under both stressed and unstressed conditions. They can promote plant growth under drought stress conditions (Lim and Kim, 2013). PGPR produce 1-aminocyclopropane-1-carboxylase (ACC) - deaminase which reduces ethylene levels in plants. Previous studies have shown enhanced stress tolerance in plants through inoculation with ACC deaminase–producing PGPR. This appears to occur since PGPR are able to keep ethylene levels from reaching levels sufficient to reduce plant growth (Glick, 2014).

5.2 Other microbe-to-plant signal molecules

Several volatile organic compounds (VOCs) and secondary metabolites secreted by bacteria can promote plant growth. It is known that polyamines play a physiological and

protective role in plants. It was reported that *B*. *megaterium* secretes spermidine and induces polyamine production in *Arabidopsis* resulting in an increase in the root biomass and elevation in photosynthetic capacity (Zhou et al., 2016). Many PGPR produce HCN that can protect the plant against deleterious microbes in the rhizosphere. VOC produced by PGPR stimulate plant growth, increase the shoot biomass and improve the plant stress resistance (Bailly and Weisskopf, 2012); (Ruzzi and Aroca, 2015).

5.3 Root exudates as plant-to-microbe signals

Root exudates are released from roots into the surrounding soil and help microbial growth and activity in the rhizosphere. The variation in root exudation (constituents, timing and amount) can manipulate the composition and the abundances of root-associated microbiota. It was reported that exudation of the signal molecules salicylic acid and jasmonic acid in the rhizosphere can be involved in the interaction between the roots and the surrounding microbes at the beginning of the colonization (Gutjahr and Paszkowski, 2009; Doornbos *et al*., 2011).

6. Indoleacetic acid (IAA)

Microbial synthesis of the phytohormone auxin (IAA) has been known for a long time. It is reported that 80% of microorganisms isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites (Patten

and Glick, 1996). Several naturally occurring auxins have been described in literature. IAA is the most recognized and most studied auxin. Literature considers auxin and IAA to be interchangeable terms. PGPR secrete IAA, which is a plant hormone that is produced in buds and young leaves through various, independent biosynthetic pathways. These pathways include: the indole-3-acetamide pathway, the indole-3-pyruvic acid pathway, the tryptamine pathway, and the indole-3-acetaldoxime pathway (Mano and Nemoto, 2012).

IAA causes a rapid increase in cell wall extensibility in young stems (Majda and Robert, 2018). IAA plays an important role in flower and leaf senescence and abscission (Lombardi *et al*., 2015).

IAA affects plant cell division, differentiation and extension, stimulates seed and tuber germination and increases the rate of xylem and root development. Moreover, IAA controls processes of vegetative growth, initiates lateral and adventitious root formation, mediates responses to light and gravity, affects photosynthesis, and stimulates resistance to stressful conditions (Spaepen *et al*., 2007). It has been known for a long time that different IAA concentrations affect the physiology of plants in different ways. Plant responses to IAA differ from one species to another; some plants species are more sensitive to IAA than others.

Tryptophan is the most important molecule that can limit the level of IAA synthesis. It is identified as the main precursor for IAA. It also plays an important role in modulating the level of IAA biosynthesis. Tryptophan stimulates IAA production, whereas anthranilate, a precursor for tryptophan, reduces IAA synthesis. By this mechanism, IAA biosynthesis is fine-tuned because tryptophan inhibits anthranilate formation by a negative feedback regulation on anthranilate synthase, resulting in an indirect induction

of IAA production (Spaepen and Vanderleyden, 2011). It was reported that large numbers of indole-3-acetic-acid (IAA) producing bacteria have been isolated from the rhizosphere of rice (Chaiharn and Lumyong, 2011), sugarcane and ground nut (Priya *et al*., 2013), wheat (Iqbal and Hasnain, 2013), sweet potato (F. Yasmin *et al*., 2009), chickpea (Joseph *et al*., 2012), tomato and carrot (Lwin *et al*., 2012). Also, it was reported that most IAA producing bacteria can promote the plant growth and development (Khan *et al*., 2016).

Many PGPR have been shown to produce IAA including *Bacillus* (Chagas *et al*., 2015), *Streptomyces* (Hariharan *et al*., 2014) and *Pseudomonas* (Malik and Sindhu, 2011). It was reported that IAA producing *Bacillus megaterium* isolated from tea rhizosphere stimulates plant growth promotion (Chakraborty *et al*., 2006). IAA producing *Pseudomonas aeruginosa* stimulate nitrogen and phosphorus uptake by chickpea (Verma *et al*., 2013).

The variation of IAA production among PGPR was documented by Prakash and Karthikeyan (2013). Ten bacterial strains isolated from *Acoruscalamus* rhizospheric soil were identified as *Pseudomonas* sp., *Azospirillum* sp., *Azotobacter* sp., *Bacillus* sp. and were then tested for IAA production. IAA production capability varied among these strains (Prakash and Karthikeyan, 2013). IAA production by *Bacillus* remains a common characteristic among rhizobacterial isolates (Agrawal and Agrawal, 2013).

IAA produced by bacteria influence many interactions between plants and bacteria. Plant growth promotion and root nodulation are stimulated by IAA. Also, IAA production by PGPB *Pseudomonas putida* GR12-2 played a major role in the growth and development of canola roots as demonstrated by an IAA-deficient mutant of this strain (Patten and Glick, 2002).The inoculation of seeds with the wild-type *P. putida* GR12-2

stimulated root growth. These were 35–50% longer than the roots from seeds treated with the IAA-deficient mutant and the roots from uninoculated seeds. In contrast, mung bean cuttings inoculated with a mutant strain (Gupta and Gopal, 2008) which produces a great amount of IAA yielded shorter roots than the control (Mayak et al., 1999). The reason of this result is the combined effect of auxin on growth promotion and the inhibition of root elongation by ethylene (Jackson, 1991). Bacterial IAA, which was incorporated by the plant, likely stimulated the activity of the ACC synthase enzyme and consequently increased the synthesis of ACC (Jackson, 1991) followed by an increase in the level of ethylene which in turn inhibited root elongation (Riov and Yang, 1989). Bacterial IAA increases root length and root surface area and as a result it provides the plant with greater access to soil nutrients. Moreover, bacterial IAA loosens plant cell walls, thereby facilitating an increasing amount of root exudation which in turn provides additional nutrients to support bacterial growth in the rhizosphere.

IAA protects the plant against several phytopathogenic bacteria by strengthening the plant defence mechanisms (Olanrewaju *et al*., 2017) . IAA produced by PGPR stimulates physiological processes in plants by altering the hormone balance in the host plant (Egamberdieva, 2009). IAA therefore controls every aspect of plant growth and development as well as defense responses (Gray, 2004). This diversity of IAA functions may be explained by its complex biosynthetic, transport and signaling pathways. Consequently, IAA produced by PGPR is identified as an effector molecule in plant–microbe interactions, in both plant growth promotion and biocontrol of phytopathogens (Zhao, 2010).

7. Streptomyces as plant growth promoting rhizobacteria

Members of the genus *Streptomyces* are Gram-positive bacteria (Barka *et al*., 2015). Most of them are known to be saprophytic soil organisms. They belong to the order *Actinomycetales,* phylum actinobacteria and the family *Streptomycetaceae* (Kämpfer *et al*., 2014). They have genomes with high GC content. They are aerobic and filamentous bacteria that produce vegetative hypha with branches that form substrate mycelium. They are spore-forming bacteria and their spores facilitate their dispersal in the environment (Wildermuth, 1970).

Streptomyces spp. can colonize the rhizoplane of the host plant. Many strains of which gain access to root tissues from the rhizosphere (Vurukonda *et al*., 2018). Some of them are known as endophytes as they colonize the inner tissues of some host plants and complete their life cycle in plant tissues (Coombs and Franco, 2003). They can survive in different environmental conditions. They are found in soils and rhizospheres of different structures and chemistry of several plant species (Ramakrishnan *et al*., 2009). They can establish beneficial plant–microbe interactions (Olanrewaju and Babalola, 2019). Certain *Streptomyces* spp. used as biofertilizers. They can directly promote plant growth and influence soil fertility by increasing the availability of nutrients, solubilizing phosphate, producing siderophores and secreting enzymes which transform complex nutrients into simple mineral forms (Olanrewaju and Babalola, 2019). They can also act as biocontrol agents in greenhouse or field trials by protecting plants against the deleterious effects of pathogenic bacteria. Hence, they enhance plant resistance against several phytopathogenic diseases following root colonization (Law *et al*., 2017).

Streptomyces spp. have been reported as PGPR in a wide range of plants including rice (Gopalakrishnan *et al*., 2013), banana (Chen *et al*., 2017) and wheat (El-Shanshoury, 2008). Different species of *Streptomyces* can stimulate plant growth by fixing atmospheric nitrogen and producing IAA (Suárez-Moreno *et al*., 2019). Previous studies showed that the culture filtrates of two different *Streptomyces* species increased significantly the shoot length and shoot fresh mass of wheat. Hormone extraction and purification showed that both species produced great amounts of phytohormones, including auxins. This result suggested that *Streptomyces* spp. produce at least one class of compounds that directly influence plant growth (Aldesuquy *et al*., 1998). It was reported that *S. rochei* IDWR19 and *S. thermolilacinus* IDRWR81 exhibited PGP activities including siderophore production , IAA synthesis, phosphate solubilization and this significantly improved growth and development of wheat cv. (Jog *et al*., 2012). Parallel to this, Franco-Correa and collaborators documented that *Streptomyces* strains isolated from soil exhibited PGPR traits including siderophore production, phosphate solubilization and N_2 fixation, and were able to promote plant growth (2010).

A variety of bioinoculants (biofertilizers) are already on the market globally. Microbial inoculants have many advantages when compared to chemical fertilizers but this through careful selection of suitable strains. Inoculants present a reduced risk to the environment and human health. The action of bioinoculants is more targeted and they are effective in small quantities. They can survive to the next season and can be used on their own or in combination. Example of biocontrol and other PGP microbial products is Arzent™: a mixture of four compatible strains of *S. hygroscopicus* tested for its ability to promote radish growth in the greenhouse. , Radish wet weight was found to be 13% greater than the untreated controls (Doumbou *et al*., 2001; Hamby and Crawford, 2000). These results demonstrated the capability of a *Streptomyces* strain to promote plant growth, independent of their well -Known potential as pathogen antagonists.

There are many other effective PGPR on the market. It was reported that under growth chamber conditions, carrot seeds treated with *S. lydicus* WYEC 108 increased carrot wet weight by 21% over those with untreated controls. *S. griseoviridis* K61 (Mycostop®) is a biocontrol agent and a PGP microbial product (Pereg and McMillan, 2015). *Streptomyces* sp. strain SB14 (Micosat F UNO, CCS Aosta Srl) was reported as plant growth promoter. Microbial consortia are also commercially available to farmers worldwide. They are used in agriculture as biofertilizers. Examples include Micosat F® (CCS Aosta srl, Aosta, Italy), a product containing three different *Streptomyces* spp.; Forge SP® (Blacksmith Bioscience, Spring, TX, USA), containing *S. nigrescens*; and Mykorrhyza soluble 30G (Glückspilze, Innsbruck, Austria), containing *S. griseus* and *S. lydicus* (Vurukonda *et al*., 2018). Bacterial consortia can be used as efficient inoculants as they could have better effects than a single strain on plants since different strains of PGPR could synergistically interact to provide the plant with more nutrients (Egamberdieva *et al*., 2016)

8. *Bacillus* as plant growth promoting rhizobacteria

The genus *Bacillus* is a member of the phylum Firmicutes and family Bacillaceae. They are Gram positive aerobic bacteria. They are commonly found in rhizosphere, bulk soil, phyllosphere and water. This genus includes 266 named species including *B. thuringiensis*, *B. cereus*, *B. subtilis* and *B*. anthracis (Koehler, 2009; Rooney *et al*., 2009).

Bacillus based biofertilizers can be used as safe alternatives to chemical fertilizers as they can enhance plant growth and yield. Many *Bacillus* strains were recorded as

PGPR (Glick,1995) and they are the main constituents of several agricultural products. The application of *Bacillus*-based fertilizers to soil can increase nutrient available in the rhizosphere, resulting in plant growth promotion. They can also act as biocontrol agents protecting the plants against several diseases and inducing defense against pests (García-Fraile *et al*., 2015). Lytic enzymes produced by *Bacillus* such as protease, glucanase, chitosanase and cellulase damage pathogenic bacteria, fungi, nematodes, viruses and pests, protecting the plant against several diseases. Plant-beneficial *Bacillus* spp. are known to associate with roots and develop biofilms to increase plant growth (Beauregard *et al*., 2013).

Bacillus isolates are spore-forming bacteria. They can survive for a long time under unfavorable environmental conditions. They protect agricultural crops faced with various stressors including heavy metal accumulation in soil, water scarcity, and salinity. *Bacillus* spp. can limit the motility of toxic ions, modulate the ionic balance and water transport in plant tissues, while controlling the pathogenic microbial population by producing siderophores and exopolysaccharides (Radhakrishnan *et al*., 2017).

Bacillus associations can stimulate plant immunity against stresses by altering stress-responsive genes, proteins, phytohormones and related metabolites (Radhakrishnan *et al*., 2017). Moreover, the synthesis of gibberellic acid, IAA, and ACC deaminase by *Bacillus* strains regulates plant intracellular phytohormone levels and can enhance stress tolerance and growth in plants (Kumar *et al*., 2011).

Bacillus isolates are effective biofertilizers, notably due to their capacity to form spores these enhance their viability in commercial formulations. *Bacillus*-based biofertilizers can also survive within a wide range of biotic and abiotic environments. Alinit is the first

commercial biofertilizer composes from *Bacillus* spp., its application resulted in a 40% increase in crop production (Kilian *et al*., 2000). Other *Bacillus*-based fertilizers that are used by farmers commercially worldwide include Rhizovital (*Bacillus amyloliquefaciens* FZB42), Serenade (*B. subtilis* QST713), Sonata® TM *(Bacillus pumilus* QST 2808) and YIB (*Bacillus* spp.) (Pereg and McMillan 2015; Brannen and Kenney, 1997; Ngugi *et al*., 2005; Cawoy *et al*., 2011).

Bacillus spp. secrete phosphatases and organic acids which have the capacity to convert inorganic phosphate to free phosphate (Radhakrishnan *et al*., 2017). Several *Bacillus* spp. release ammonia from nitrogenous organic matter. It was reported that some *Bacillus* strains have the *nifH* gene and produce nitrogenase that can fix atmospheric N_2 and provide it to plants to stimulate plant growth by delaying senescence (Masclaux-Daubresse *et al*., 2010) .

In the presence of tryptophan *Bacillus* spp. can produce phytohormones such as IAA, gibberellins and cytokinins that stimulate plant growth and development. *Bacillus* spp. secrete ACC deaminase which inhibits ethylene synthesis in plants thus promoting plant growth. ACC deaminase converts ACC into ammonia and ketobutyrate and the interaction between ACC deaminase and IAA facilitates the reduction of ethylene in the plant resultantly promote the plant growth (Honma and Shimomura, 1978); Olanrewaju *et al*., 2017) .

It was reported that *Bacillus* strains can act as promoting bacteria for several plant species and having PGP traits as in soybean ,wheat (Akinrinlola *et al*., 2018) and rice (Win *et al*., 2018). It was reported that *Bacillus* sp. PSB10 significantly improved growth,

nodulation, chlorophyll, seed yield and grain protein in chickpea (Wani and Khan, 2010).

In addition to the PGP ability of *Bacillus* strains, they can be used as biocontrol agents to protect plants against several diseases. It was reported that *B. licheniformis* MH48 was able to protect *Camellia oleifera* against foliar fungal diseases by producing the lytic enzymes chitinase and β-1,3-glucanase. This strain was also able to increase the total nitrogen and phosphorus contents in soils through N_2-fixation and P-solubilization, thereby promoting the growth of *Camellia oleifera* (Won et al., 2019).

It was reported that *B*. *subtilis* BHUJP-H1, *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 exhibited several PGPR traits and their mixed combination promoted the growth of *Vigna radiata* (Verma *et al*., 2018).

9. Consortium of plant growth-promoting rhizobacteria

A bacterial consortium can be defined as a combination of two or more bacterial strains. The concept of consortium was first reported by Johannes Reinke in 1872. The concept is to use a bacterial combination of PGPR strains to shift microbiological equilibria and promote plant growth, production, nutrient uptake, and protection. Individual PGPR strains sometimes show inconsistent results under field conditions while bacterial consortia of PGPR have been shown to provide more consistency (Figueiredo *et al*., 2011, Belimov *et al*., 1995). Pandey and collaborators (2012) reported that each of the

strains composing a consortium competes not only with the others for rhizospheric colonization but they are also functionally complementary for the promotion of plant growth. They may interact synergistically to improve the availability of nutrients, produce siderophores, fix atmospheric nitrogen, help in nodulation, produce enzymes, stimulate the ISR and produce IAA (Panwar *et al*., 2014). Various strategies can be considered in formulation and designing of effective bacterial consortium. Understanding of the of interactions between strains is required. Previous studies indicated that some strain combinations allow bacteria to use different strategies that enables them to interact with each other synergistically, remove inhibitory products, and provide nutrients. They can stimulate each other through physical and biochemical activities, this can stimulate some aspects of their physiology, and improve the plant growth (Bashan, 1998). It was reported that a PGPR consortium consisting of *Pseudomonas putida* KT2440, *Sphingomonas* sp. OF178, *Azospirillum brasilense* Sp7 and *Acinetobacter* sp. EMM02 improved maize growth and the individual strains composing it exhibited PGP traits including siderophore production, IAA production and phosphate solubilization (Molina-Romero *et al*., 2017).

Antagonistic relationship studies should be performed during the design of bacterial consortia. Indeed, the compatibility of strains in combined inoculations is important to avoid antagonism and promote plant growth. For example, Santiago and collaborators (2017) recently showed that the co-inoculation of the compatible *Streptomyces* sp. R170 with *Sphingomonas* sp. T168 or *Methylibium* sp. R182 enhanced the growth of potato seedlings while the co-inoculation of the incompatible *Streptomyces* sp. R181 with *Sphingomonas* sp. T168 or *Methylibium* sp. R182 did not significantly increase the plant growth compared to controls .

The use of multiple strains in a bacterial consortium to pursue multiple benefits can also enhance inoculum adaptation in specific ecological niches. Using bacterial consortia

may have superior effects to promote plant growth than single strains possibly by removing inhibitory products and improving mitigation to external stresses (Molina-Romero *et al*., 2017). Moreover, may be some substrates in the soil that are partially degraded by the first strain composing the consortium are completely degraded by the second strain composing it (Puentes-Téllez and Falcao Salles, 2018); making it more utilizable by the plant. In addition to, the inoculation of an individual strain could be not active in all types of agricultural ecosystems and different kinds of soils, this may lead to insufficient colonization, limited tolerance to environmental changes (Raupach and Kloepper, 1998). Similarly, it was reported that co-inoculation of two PGPR, *Enterobacter* sp. and *Pseudomonas* sp., resulted in better survival of these strains, as compared to individual (Neyra *et al*., 1995).

It was reported that the mixed culture of *Pseudomonas* and *Bacillus* increased significantly seedling growth of wheat under field experiments (van Elsas *et al*., 1986). Inoculation with a mixture of *Bacillus licheniformis* and *Phyllobacterium* sp and a mixture of two *Azospirillum brasilense* strains increased significantly plant height and dry weight of oilseed (Bashan *et al*., 2000). A previous study also reported that the co-inoculation of wheat seedlings with *Azospirillum* sp. and *Azotobacter* sp. increased significantly seedling growth and the concentrations of IAA, P, Mg, and N; and total soluble sugars in plant tissues (El-Shanshoury,1995). The combined inoculation of *Methylobacterium oryzae* with *A. brasilense* and *Burkholderia pyrrocinia* was reported to have a positive effect on nutrient uptake and growth of tomato, red pepper, and rice plants (Madhaiyan et al., 2010). Moreover, under drought stress conditions the combined application of three PGPR was more efficient than single inoculations in promoting the growth and nodulation of common bean (Figueiredo *et al*., 2008).

Finally PGPR consortia can also act as biocontrol agents suppress diseases- causing microbes in a wide range of agricultural crops (Sudharani *et al*., 2014; Thakkar and Saraf, 2015). Wang and collaborators (2012) reported that a combination of three

PGPR strains (*Bacillus cereus* AR156, *Bacillus subtilis* SM21, and *Serratia* sp. XY21) decreased wilting symptoms in cucumber plants.

10.1 General objective

The aim of this study was to establish for the first time PGPR consortia formed with actinobacterial and *Bacillus* strains with the aim of exploiting the spore-forming character of *Streptomyces* and *Bacillus* spp., which could enhance the viability of cells in future, commercially formulated products. Moreover, the combination of *Streptomyces* and *Bacillus* strains could provide significant benefits to the plant, greater than those by each strain alone.

10.2 Specific objectives

1) Screen a collection of actinobacteria and *Bacillus* strains for IAA production.

2) Determine the growth-promoting potential of the most interesting auxin producing actinobacteria and *Bacillus* isolates by inoculating them onto *Lemna minor*.

3) Investigate the compatibility (non antagonism) of the most interesting actinobacterial strains together and with PGPR *Bacillus* strains.

4) Determine the effect of selected bacterial consortia on growth promotion of *L. minor*.

5) Determine the effect of selected actinobacterial and *Bacillus* isolate (alone or in combination) on growth promotion of lettuce.

6) Determine of the taxonomic identity of the strains composing the selected consortia.

CHAPTER 2

LEMNA MINOR AND LETTUCE GROWTH PROMOTION USING AUXIN PRODUCING *STREPTOMYCES* AND *BACILLUS* STRAINS ALONE OR IN CONSORTIA

2.1. Preamble

Using chemical fertilizers to promote plant growth have negative impacts on the environment and human health This has inspired interest in using plant growth promoting rhizobacteria (PGPR) as biofertilizers. PGPR promote plant growth by different mechanisms such as indole acetic acid (IAA) production. PGPR consortia have been shown to provide more consistency under field conditions compared to individual PGPR strains that sometimes show inconsistent results. The aim of this project was to establish PGPR consortia from actinobacteria and *Bacillus* isolates. To our knowledge, this is the first time reporting bacterial consortia composed of actinobacterial and *Bacillus* strains promoting plant growth. The scientific value of this work lies in exploiting the spore-forming character of *Streptomyces* and *Bacillus* spp., which could enhance the viability of cells in future commercially formulated products. Moreover, the combination of *Bacillus* and *Streptomyces* strains could provide significant beneficial activities for the plant, greater than the activities provided by the strains alone.

2.2. Title of the article: « *Lemna minor* and lettuce growth promotion using auxin producing *Streptomyces* and *Bacillus* strains, alone or in consortia ».

Lisa Emad, Pascale Beauregard and Carole Beaulieu are the authors of the article. The contribution of each author in the article is as follows: Laboratory work, development and adaptation of techniques and the whole analysis were done by Lisa Emad. The work was supervised by Carole Beaulieu and Pascale Beauregard.

2.3 Résumé

Les rhizobactéries qui favorisent la croissance des plantes (RFCP) peuvent être utilisées comme biofertilisants car elles favorisent la croissance des plantes par différents mécanismes tels que la production d'auxine. Cependant, les souches de RFCP individuelles montrent parfois des résultats variables dans des conditions réelles, alors que les consortiums de RFCP offrent une plus grande fiabilité. Le but de cette étude était d'établir pour la première fois des consortiums de RFCP formés avec des souches d'actinobactéries et de *Bacillus*. Les membres de ces groupes ont été criblés pour la production d'acide indole-3-acétique (IAA). La capacité des souches les plus productives en IAA à favoriser la croissance de la plante modèle *Lemna minor* a été testée. Respectivement, 73% et 11% des souches d'actinobactéries et de *Bacillus* sélectionnées produisant de l'IAA ont favorisé la croissance de *L. minor*. La compatibilité entre les souches sélectionnées a été déterminée par la technique de superposition de double gélose. Il n'a pas été possible de former des consortiums contenant plus de trois souches en raison d'un antagonisme entre les souches. Quatorze consortiums ont été testés et sept d'entre eux ont favorisé la croissance de *L. minor*. La capacité d'une combinaison d'isolats compatibles à promouvoir les nombres de frondes de *L. minor* s'est révélée égale ou inférieure à la capacité des souches simples composant le consortium. Les consortiums A et E ont également augmenté la croissance de la laitue, indiquant que *L. minor* est une bonne plante modèle pour le dépistage de RFCP. La capacité du consortium A à promouvoir la croissance de la laitue était égale à celle de la souche unique JW 239, tandis qu'une synergie était observée entre les membres du consortium E, ce qui suscitait de l'intérêt pour l'application sur le terrain.

Mots clés : Consortia, *Bacillus*, *Streptomyces,* acide indole-3-acétique, *Lemna minor*, laitue.

2.4 *Lemna minor* and lettuce growth promotion using auxin producing *Streptomyces* and *Bacillus* strains alone or in consortia.

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Abstract

Plant growth promoting rhizobacteria (PGPR) can be used as biofertilizers since they promote plant growth by different mechanisms such as auxin production. However, individual PGPR strains sometimes show inconsistent results under field conditions while PGPR consortia have been shown to be more reliable. The aim of this study was to establish for the first time PGPR consortia formed with actinobacterial and *Bacillus* strains. Members of these groups were screened for indole-3-acetic acid (IAA) production. The capacity of the highest IAA producing strains to promote the growth of the model plant *Lemna minor* was tested. Respectively, 73% and 11% of the selected IAA producing actinobacterial and *Bacillus* strains promoted *L. minor* growth. Compatibility between the selected strains was determined by double agar overlay technique. It was not possible to form consortia containing more than three strains due to antagonism between strains. Fourteen consortia were tested and seven of them promoted *L. minor* growth. The ability of a combination of compatible isolates to promote *L. minor* frond numbers was found to be equal or lower than the ability of the single strains composing the consortium. Consortia A and E also promoted lettuce growth, indicating that *L. minor* is a good model plant to screen PGPR. Capacity of consortium A to promote lettuce growth was equal to the single strain JW 239 while a synergy was observed between members of consortium E, suggesting its potential for further studies that would be conducted in the field.

Key words: Consortia*, Bacillus, Streptomyces,* indole-3-acetic acid, *Lemna minor*, lettuce.

Introduction

The world faces a food security problem owing to the global overpopulation which leads to increase the use of the chemical fertilizers and thus increase agriculture productivity to meet the food demand. Chemical fertilizers have negative impacts on the environment and on human health (Roberts, 2009). This has inspired interest in using plant growth promoting rhizobacteria (PGPR) as biofertilizers as they are free-living bacteria of agricultural importance that colonize the rhizosphere (Sindhu et al., 1999). They establish associations with plants and improve soil quality without polluting environment, ensuring sustainable crop production at low cost.

PGPRs can be found in several bacterial species such as *Pseudomonas*, *Bacillus*, *Azospirillum*, *Azotobacter*, *Streptomyces*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Flavobacterium*, *Burkholderia*, *Bradyrhizobium*, *Mesorhizobium*, *Rhodococcus* and *Serratia*, which enhance plant growth and yield production (Verma *et al*., 2019). PGPR have been shown to promote and stimulate plant growth and development by colonizing the roots (Sindhu et al., 1999). PGPR can perform by different mechanisms such as biological N_2 fixation, phosphate solubilization, phytohormone production (e.g. auxins) and increasing iron nutrition through iron-chelating siderophores (Ahemad and Kibret, 2014). In addition, PGPR could indirectly promote plant growth by the elicitation of induced systemic resistance and production of antimicrobial compounds which protect the plant against deleterious microorganisms and populations of root pathogens. They can also facilitate the uptake and availability of nutrients from the rhizosphere, thus benefiting the plant growth (Figueiredo et al., 2016).

Auxin, indole-3-acetic acid (IAA), is known for its importance as a plant growth hormone (Yasmin et al., 2009). Studies have demonstrated that auxin production by PGPR can promote the plant growth by changing the hormonal content of the host plant (Backer et al., 2018). Auxin can control important physiological activities in plants and participates in all stages of plant growth from embryo to adult reproductive plant. It is thus responsible for most of the developmental patterns in plant (Moller and Weijers, 2009) such as cell enlargement and division, tissue differentiation and response to light and gravity (Takatsuka and Umeda, 2014).

Streptomyces spp. can establish beneficial plant–microbe interactions (Olanrewaju and Babalola, 2019). *Streptomyces* spp. have been reported as PGPR as they directly promote plant growth including rice (Gopalakrishnan et al., 2013), banana (Chen et al., 2017) and wheat (El-Shanshoury, 2008). *S. cameroonensis* strain JJY4^{T} was reported to exhibit plant growth promoting (PGP) traits including the solubilization of inorganic phosphate, the production of siderophores, and indole-3-acetic acid, and ACC deaminase activity. Consequently, in planta assays performed on cocoa plantlets confirmed that strain JJY4^{T} strongly promoted plant growth and protected against the host plant *Phytophthora megakarya* (Boudjeko et al., 2017). There are effective *Streptomyces* strains commercially available including *S. griseoviridis* K61 (Mycostop®) is a biocontrol agent and a PGP microbial product (Pereg and McMillan, 2015).

Bacillus based biofertilizers can be used as safe alternatives to chemical fertilizers as they can enhance plant growth and yield. Plant-beneficial *Bacillus* spp. are known to associate with roots and develop biofilms to increase plant growth (Beauregard et al., 2013). Many *Bacillus* strains were recorded as PGPR (Glick, 1995). It was documented that the *Bacillus* sp. B55 was able to colonize the endosphere and rhizoplane of

Nicotiana attenuate plant and promoting its growth by exhibiting several PGP activities including IAA production, phosphate solubilization and ACC deaminase production (Meldau et al., 2012). Moreover, other *Bacillus*-based fertilizers that are used by farmers are commercially available worldwide including YieldShield® (*B. pumilus* GB34), Quantum-400 (*B. subtilis* GB03) and Kodiak (*Bacillus subtilis* GB03) (Pereg and McMillan, 2015; Brannen and Kenney; 1997, Ngugi et al., 2005; Cawoy et al., 2011).

Plant models used in the present study were *Lemna minor*, as well as common lettuce. *Lemna minor* can be used as animal fodder (Sońta et al., 2019) and organic fertilizer because of its high starch content. Moreover, it is a low-cost wastewater treatment system as it can remove heavy metals and other pollutants from the water. Also, it is a promising feed stock for biofuel production because of its starch rich content which help in obtaining larger yield of biofuel ethanol (Xu et al., 2012). Lettuce is one of the top ten vegetables consumed worldwide and its known by its economic importance in Canada.

Using a consortium to promote plant growth may have better effect than using single strains because of the combination of beneficial activities provided by various PGPR, leading to a potential synergistic interaction between the strains. For example, a combination of strains could facilitate nutrient acquisition of host plants or improve their resistance to environmental stress. It was reported that the effect of inoculation of a consortium of several strains have shown better results than the inoculation of a single strain on plant growth promotion under field conditions (Figueiredo et al., 2011), (Belimov et al., 1995). Also, synergistic interaction was observed upon the combined inoculation of *Azotobacter armeniacus* and *Azotobacter nigricans* that increased rice yield but no effect was observed upon the single inoculation of each of them (Piao et al., 2005). A synergistic PGPR ability was observed on red, pepper and tomato between the IAA producing PGPR *Bacillus subtilis* AH18 and *Bacillus licheniforims*

K11. Both strains produced IAA, antifungal β-glucannase, siderophores and were capable of solubilizing insoluble phosphates (Lim and Kim, 2009). The potential of *Streptomyces* spp (alone or in consortia) to promote plant growth has previously been reported under greenhouse and field conditions, and these inocula are commercially available (Pereg and McMillan, 2015; Vurukonda et al., 2018; Boudjeko et al., 2017; Gopalakrishnan et al., 2013; Jog et al., 2012)

In this study, actinobacterial strains and *Bacillus* strains were characterized for IAA production and antagonism assay. The selected IAA-producing strains were tested for their capacity to promote growth in model plant species *L. minor* and lettuce, alone or in a bacterial consortium. Ultimately, this investigation was intended to contribute to the development of sustainable practices in agriculture.

Materials and methods

Bacterial strains

A collection of 302 actinobacterial strains isolated from the soil and rhizosphere in Quebec, Canada was readily available and used in this study. Additionally, 12 *Bacillus* strains were isolated from lettuce grown in Quebec as follows. Lettuce leaves or roots were collected and washed with distilled water. These tissues were placed in sterile Stomacher® bags containing 100 ml of 0.85% NaCl and were diced using the Bag Mixer® (400 P lab blender, France) for 2 min. Fifteen ml of the solution were then transferred to sterilized tubes and subjected to heating for 10 min at 70°C. Each sample (1 ml) was spread on nutrient agar plates containing cycloheximide (50 mg/l). These

plates were incubated at 30°C for 24 h, single colonies were then streaked on nutrient agar plates. The taxonomic identity of the purified colonies was revealed by sequencing of the *gyrA* gene (see below). *Bacillus* isolates were resuspended in glycerol 50% with Lennox broth (Wisent Inc.) and this suspension was stored at −80°C for long-term preservation.

Auxin production by actinobacterial and *Bacillus* strains

Spore suspension (10 µl) of each of the 302 actinobacterial strains and bacterial suspension (10 ul) of each of the 12 *Bacillus* isolates were streaked on YME (4 g/l of glucose, 4 g/l of yeast extract, 10 g/l of malt extract, and 15 g/l agar) and Nutrient Agar (EMD Millipore Corporation) plates, respectively. Actinobacteria and *Bacillus* strains were incubated at 30°C for 3 days and 24 h, respectively. One loopful of this bacterial inoculum was taken and inoculated in 96-well plates containing minimal medium (KH_2PO_4 0.5 g, $MgSO_4$ $7H_2O$ 0.2 g, $(NH_4)_2SO_4$ 0.5 g, $FeSO_4$ $7H_2O$ 0.01 g in 1 l distilled water) supplemented with 2.5 mM filter-sterilized tryptophan and starch 0.5%. After an incubation period of 6 days in a rotary shaker (250 rpm) at 30°C, auxin production was detected using the Salkowski's method (Ehmann, 1977). The experiment was performed in triplicate and optical density (OD) was recorded at 535 nm. The actinobacterial and *Bacillus* strains exhibiting an optical density ranging from 0.10 to 0.44 were kept for plant growth promotion assay on *L. minor*.

Antagonism assay between various auxin-producing strains

The double agar overlay method was used to test the antagonistic activity between selected auxin-producing strains (Table 2) (Dopazo et al., 1988). Selected strains were grown on the center of YME or Nutrient Agar plates by spotting 10 µl spore suspension of each individual actinobacterium or 20 µl bacterial suspension of each *Bacillus* strain, respectively, respecting the incubation period 5 days for the actinobacterial strains or 24 h for *Bacillus* strains at 30°C. A stationary phase culture broth (100 µl) of the actinobacteria or *Bacillus* was diluted in 3 ml of YME or nutrient broth supplemented with 3 g/l agar respectively then poured immediately over the actinobacteria or *Bacillus* colony on the agar plates. The plates were incubated at 30°C for 3 days and the antagonism between the strains was detected by the appearance of a growth inhibition zone around the colonies on the center. Only the actinobacteria or *Bacillus* strains that show no antagonism to each other were selected for the construction of the bacterial consortia.

Inoculum preparation for plant growth promotion assays

A spore suspension (10 µl) of each actinobacterial strain and a bacterial suspension (20 µl) of each *Bacillus* isolate were inoculated in 125 ml flasks containing 50 ml of J medium (Kieser et al., 2000). After an incubation period of 3 days in a rotary shaker (225 rpm at 30°C), the bacteria were harvested by centrifugation for 10 min (3,500 *g*) and resuspended in NaCl 0.85% (rinsing). Bacterial cultures were centrifuged again to make standard bacterial inoculum by diluting the pellet in five volumes of NaCl 0.85%. For each actinobacterial strain, the concentration 1× was 7.3×10^6 CFU/ml while it corresponded to 9×10^6 CFU/ml for each *Bacillus* strain.

Growth promotion assay on *Lemna minor*

Aseptic *L. minor* was propagated in 225 ml of modified Hoagland's nutrient solution (Langlois et al., 2003) adjusted to pH 5.8 using NaOH in a growth chamber at 24°C under illumination provided by white fluorescent light and a day/night cycle of 16/8 h.

Each individual actinobacterial and *Bacillus* strain was inoculated into Petri dishes containing 25 ml of modified Hoagland solution with no sucrose and five fronds of *L. minor*. Each individual actinobacterial strain was used at three different concentrations (1×, 2× and 4×) where 1× was 7.3 x 10^6 CFU/ml while each individual *Bacillus* strain was used at different concentrations (4×, 8×, 16×) where 1× was 9 x 10^6 CFU/ml. *Bacillus* strain R10 was used at one additional concentration (1×). Bacteria and fronds were then incubated for 10 days in a growth chamber at 24°C/16°C (day/night), with a photoperiod of 16/8 h (day/night). The growth of *L. minor* was estimated by determining the frond numbers after 5, 8, and 10 days. Any visible, protruding bud was counted to avoid individual bias (Wang, 1990). At day 10, the growth of *L. minor* was also estimated by determining the dry weight of the plants (Radić et al., 2010). Uninoculated culture media containing fronds were used as a negative control.

Growth assay was also performed with bacterial consortia. In this case, the bacterial concentration that was used for each individual strain composing the consortia was the lowest concentration that promoted the growth of *L. minor* after 10 days. The experiment was done in triplicate. The relative growth was measured for comparing all the treatments relative to the control. The one-sample *t*-test was used for determination of the treatments which are statistically different relatively to the control at $P < 0.05$. All these statistics were done using the Statistics 9 software.

Growth promotion assay on lettuce

Consortia A and E (Table 3), as well as each single strain composing both consortia, were evaluated for their capacity to promote the growth of lettuce. Seeds of the green leaf lettuce (*Lactuca sativa* L.) were germinated in a sterilized mixture of AGRO MIX® (FafardAGRO MIX® Soil Mix for Seedlings and Sprouts) and vermiculite (10:1) for 2 weeks in a growth chamber at 24°C/16°C (day/night) and with a photoperiod of 16/8 h (day/night) and a relative humidity of 90%. The lettuce seedlings were transferred to the same growth substrate (one seedling/pot) and kept under the same environmental conditions. After 4 weeks, the bacterial inoculum (10× and 100×) was added to the pots while uninoculated saline was added to the control pots.

Twenty-five days after inoculation, the plants were harvested and dried at 65°C for 3 days. The relative growth was measured by comparing a treatment relatively to the control. Five replicates were done from each treatment and the experience was repeated three times in a completely randomized design.

The one-sample t-test was used for determination of the treatment which is statistically different considering the P-value to be $P < 0.05$. Then the data were subjected to Analysis of Variance (ANOVA) to determine the significance of variances among the treatments at confident interval 95%. Comparisons of treatment means were accomplished by least significance difference (LSD) test. All statistical analyses were performed using Statistics 9 software.

Determination of the taxonomic identity of the strains composing the selected consortia

The genomic DNA of the actinobacterial strains composing the selected consortia that promoted *L. minor* growth was extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer instructions. From each sample of extracted DNA, the gene encoding 16S rRNA was amplified by PCR with primers BSF-8/20 (5'-AGAGTTTGATCCTGGCTCAG-3') and BSR-1541/20 (5'-AAGGAGGTGATCCAGCCGCA-3'), which amplify most of the full length of the 16S rRNA gene. PCR reactions were carried out in a final volume of 30 µl containing 1.5 µl (10 µM) of each primer, 3 µl of 10× buffer, 0.75 µl of 10 mM dNTP, 0.15 µl of Taq DNA polymerase (0.5U). The PCR cycling conditions were as follows: an initial pre-denaturing step at 95°C for 30 s, 35 cycles at 95°C for 20 s, 55°C for 30 s, 68°C for 1 min 10 s and a final extension step at 68°C for 5 min using a thermal cycler (Eppendorf® Master cycler Gradient, Mississauga, ON). PCR amplification products were sequenced at the Plateforme de séquençage et génotypage des génomes (Centre de recherche du CHU, Quebec City, QC). The obtained sequences were analyzed using Clustal Omega software (Sievers et al., 2011) and compared to sequences from the GenBank database using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST). *Bacillus* strain R10 was identified by amplifying the *gyrA* gene using primers 5'-CAGTCAGGAAATGCGTACGTCCTT-3'and 5'-GCCAGCAGCCGCGGTAA -3'.

Results

Isolation and screening of bacterial strains for IAA production

A collection of 302 actinobacterial and the 12 *Bacillus* strains isolated in this work were screened for IAA production by using Salkowski's reagent which detect the presence of the indole ring of IAA. IAA production in the culture medium was evident by the appearance of pink colour. The colorimetric technique showed that actinobacteria differed in their ability to produce auxin in the growth medium used here as 88 of them (29%) showed positive reaction to the Salkowski's test (Supplementary TableS1). The 26 strains that showed the highest optical density ranging from 0.099 to 0.44 (Table 1) were kept for plant growth promotion assay on *L. minor*. Nine out of the 12 *Bacillus* isolates, whose optical density ranged from 0.099 to 0.53, showed positive reaction to the Salkowski's test (Supplementary TableS1) and were therefore selected for the growth promotion assay on *L. minor*.

Table 1. Effect of individual actinobacterial strains on *L. minor* growth.

Strain	The relative growth based on the frond numbers of *L. minor* [a]									The relative growth based on the dry weight of *L. minor* [b]		
	Incubation period									Incubation period		
	5 days			8 days			10 days			10 days		
	(1×) [c]	(2X)	(4X)	(1X)	(2X)	(4X)	(1X)	(2X)	(4X)	(1X)	(2X)	(4X)
JW 239	1.25*	1.53*	1.17	1.47*	1.79*	1.32*	1.41	1.76*	1.27*	1.06*	1.17*	0.98
N106	1.28*	1.28*	1.17	1.52*	1.23*	1.02	1.37*	1.16	0.90	0.98	0.98	0.96
EF-24	1.29*	0.96	1.32*	1.68*	1.32	1.48*	2.04*	1.28	1.63*	1.30*	1.03	1.09*
EF-38	1.32*	1.32*	0.92	1.77*	1.82*	1.43*	1.64*	2.02*	1.56*	1.22*	1.27*	1.03
EF-137	1.37*	1.51*	0.92	1.42*	1.46	0.82	1.61*	1.34*	0.88	1.08	1.08	0.97
EF-116	1.45*	1.62*	1.32*	1.42	1.85*	1.38*	1.45*	1.85*	1.51*	1.06*	1.06	1.04
EF-133	1.12	1.29*	1.21	1.42*	1.46*	0.88	1.22	1.37*	0.94	1.04	1.10*	0.97
EF-16	1.13	1.31*	1.48*	1.37*	1.41*	1.69*	1.34	1.53*	1.24*	1.06	1.31*	1.07*
GRA-23	0.89	1.33*	0.92	0.83*	1.26*	0.93	0.91	1.26*	1.08	1.02	1.36*	1.02
EF-9	1.16	1.45*	1.25	1.18	1.43*	1.29*	1.03	1.43*	1.21	1.01	0.98	1.01
EF-100	0.92	1.10	1.25*	1.19	1.26*	1.11	1.14	1.18	1.27	1.11*	1.00	1.03
D-1	1.11	1.11	1.37*	1.44	1.38*	1.55*	1.40	1.18*	1.48*	1.32*	0.84	1.35*
ZX7puk6403	1.04	1.12	1.41*	1.18	1.25	1.31*	1.09	1.12	1.34	1.01	1.00	1.03
JJy4	1.18	1.22	1.14	1.26*	1.38	1.32*	1.23	1.18	1.22	1.17	1.16	1.17
EF-136	0.96	1.07	1.03	1.20*	1.23*	1.23	1.09	1.07*	1.34	1.03	1.01	1.09
MG1655	1.14	1.14	1.03	1.35*	1.38*	1.41	1.31	1.32	1.34	1.02	1.17	1.25
EF-21	1.22	1.04	1.22	1.41*	1.16	1.21	1.61*	1.19	0.88	1.73*	1.06	1.01
EF-43	1.12	0.96	0.71*	1.45	1.34	0.67*	1.28	1.11	0.68*	1.01	0.98	0.95*
NC-2013	1.03	1.11	1.03	1.12	1.17	1.06	1.20	1.17*	1.01	1.27	1.25	0.95
C-8	1.01	1.07	1.03	0.93	1.14	1.03	1.02	1.14	0.86	1.02	1.09	1.01
ESS2368	1.07	1.07	0.96	1.08	0.97	0.88	0.97	0.95	0.90*	0.95	0.98	0.95
GRA12	1.14	1.03	1.07	1.06	1.06	1.03	1.26	1.08	0.91	1.36*	1.11*	1.13*
EF-40	0.93	1.01	0.84*	0.91	1.35	0.92	0.91	1.13	0.89	0.90*	0.91*	0.95*
Pru16	1.03	0.88	0.77	1.16	0.77	0.83*	1.17	0.85	0.79	1.14	0.92	0.92
Vic8	1.01	0.87	1.12	1.02	1.01	1.03	0.96	0.93	0.87	1.02	1.03	1.02
EF-76	0.96	0.85	0.77	1.16	1.06	0.67	1.23*	1.05	0.64*	1.13*	1.01	0.73

[a] Relative growth represented by the mean of fronds of *L. minor* in each treatment divided by the mean of fronds in the uninoculated control treatment.
[b] Relative growth represented by the mean of the dry weight of *L. minor* in each treatment divided by the mean of the dry weight of the uninoculated control treatment.
[c] (1×, 2× and 4×) corresponded to the concentration of the actinobacteria added to the culture medium where 1× was 7.3 x 10^6 CFU/ml.
*Refers to the value which is statistically different from the control (one-sample *t*-test, $P < 0.05$).

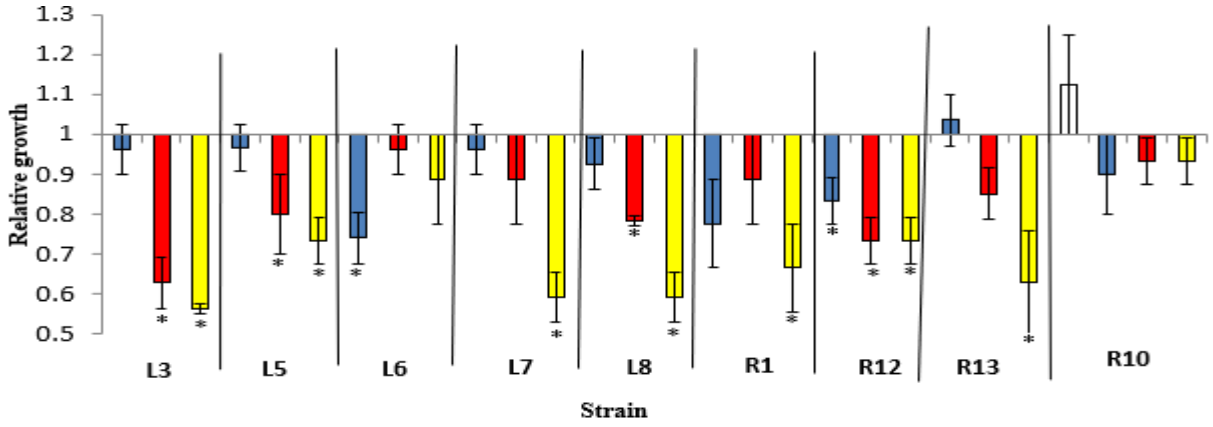
Growth promotion assay on *Lemna minor*

Of the selected 26-auxin producing actinobacterial strains, 19 promoted *L. minor* growth, at least at one of the concentrations tested, after either 5, 8 or 10 days. Their growth promotion ranged from 18% to 104% relative to the control based on the increase in the number of *L. minor* fronds (Table 1); while 14 actinobacterial strains out of the 26 increased *L. minor* dry weight from 6% to 73% relatively to the control at different concentrations (1×, 2×, or 4×). Six actinobacterial strains showed negative effect on the number of fronds and dry weight of *L. minor* at certain concentrations (Table 1).

In contrast, only the *Bacillus* strain, R10, significantly promoted the growth after 10 days at concentration 8× (Fig. 1). This strain was then also tested at a lower concentration (1×). At this concentration, it promoted *L. minor* growth after 8 and 10 days (Fig. 1). The number of fronds was promoted from 13% to 18% relatively to the control and that

corresponded to an increase in the dry weight from 6% to 36%. Most *Bacillus* strains inhibited *L. minor* growth (Fig. 1). This growth inhibition corresponded to a reduction in dry weight from 10% to 27% relative to the control at different concentrations (4×, 8×, or 16×).

A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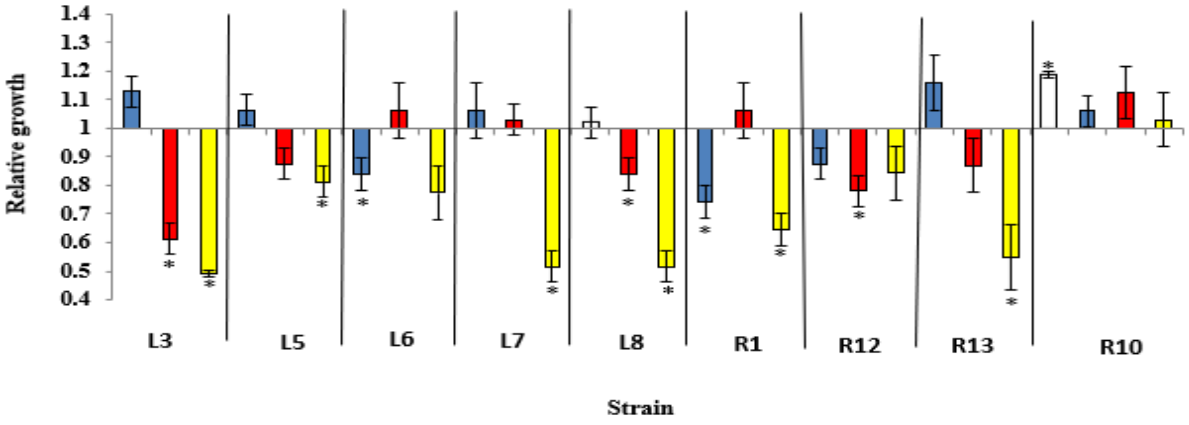
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
L3
L5
L6
L7
L8
R1
R12
R13
R10
Strain

B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1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
L3
L5
L6
L7
L8
R1
R12
R13
R10
Strain

C

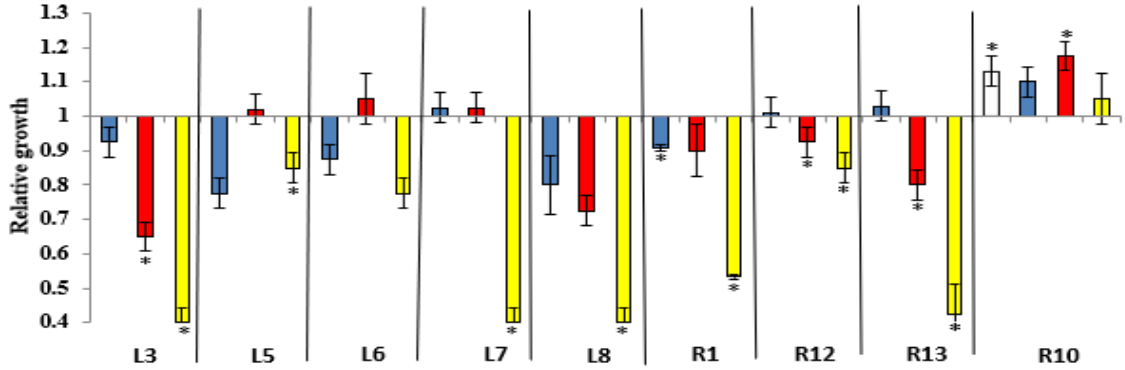
Relative growth
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
L3
L5
L6
L7
L8
R1
R12
R13
R10

Figure 1. Relative growth based on the number of fronds of *L. minor* after 5 (A), 8 (B) and 10 (C) days. White, blue, red and yellow bars represent the inoculation of the *Bacillus* strains in the culture medium at the concentrations (1×, 4×, 8× and 16× respectively; where 1× = 9 x 10^6 CFU/ml). *Refers to the value which is statistically different from the control (one sample *t*-test, $P < 0.05$).

Antagonism assays for the selection of compatible bacteria

The double agar overlay technique was used for detecting the antagonism between the eight actinobacterial strains (which showed the highest ability to promote *Lemna minor* growth) and the *Bacillus* sp. strain R10 (which promoted *Lemna minor* growth) (Table 2.). A relatively low fraction (19%) of the tested bacterial combinations were compatible. Consequently, only 14 bacterial consortia were constructed with strains showing no antagonism (Table 3) and tested for their capacity to promote *L. minor* growth. It was not possible to make a consortium containing more than three strains because of antagonism patterns.

Table 2. Antagonism assay between the selected bacterial strains

	Actinobacterial strain								*Bacillus* strain
Center / Overlay	EF-116	EF-16	E-F-24	EF-38	EF-21	Jw239	EF-133	EF-9	R 10
EF-116		+ [a]	– [b]	+	+	−	−	+	−
EF-16	+		−	+	+	+	−	+	+
EF-24	−	−		+	−	−	−	+	+
EF-38	+	−	−		+	+	−	+	+
EF-21	+	+	−	−		−	−	+	−
JW 239	+	+	+	+	+		−	−	+
EF1-33	+	+	+	+	−	−		−	−
EF-9	−	+	−	+	−	−	−		−
R10	−	−	−	+	−	−	−	−	

[a] + refers to the presence of a clear zone (presence of antagonism).

[b] − absence of a clear zone (no antagonism).

The fourteen bacterial consortia were tested for promotion of *L. minor* growth (Table 3). Ten consortia increased *L. minor* dry weight from 41% to 172% relatively to the control. This corresponded to a promotion in the number of fronds of *L. minor* from 28% to 70% after 10 days by seven consortia (Table 3).

Table 3. Growth promotion assay of the 14 selected bacterial consortia on *Lemna minor*

Bacterial consortium	Strains (concentration)	Relative growth (fronds) [a]	Relative growth (dry weight) [b]
A	JW 239 (2×)[c], EF-133 (2×)	1.71*	2.72*
B	EF-16 (1×)[d], EF-24 (1×)	1.63*	1.26
C	EF-133 (2×), JW 239 (2×), EF-9 (2X)	1.62*	2.6*
D	EF-116 (1×), EF-24 (×)	1.58*	1.53*
E	EF-9 (2×), R10 (1×) [e]	1.58*	1.72*
F	R10 (1×), EF-21(1×), EF-133 (2×)	1.42*	1.51*
G	R10 (1×), EF-21(×)	1.28*	1.41*
H	EF-21(×), EF-133 (2×)	1.01	1.22
I	EF-21(×), EF-24 (×)	1.23	1.31
J	R10 (1×), EF-133 (2×)	1.38	1.41*
K	R10 (1×), EF-9 (2×) EF-133 (2×)	1.38	1.47*
L	EF-9 (2×), EF-133 (2×)	1.46	1.64*
M	JW 239 (2×), EF-9 (2×)	1.63	1.83*
N	R10 (1×), EF-116 (×)	1.45	1.24

[a] Relative growth after 10 days represented by the mean number of *L. minor* fronds in

each treatment divided by the mean number of fronds in the uninoculated control treatment.

[b] The relative growth represented by the mean of the dry weight of *L. minor* in each treatment divided by the mean of the dry weight of the uninoculated control treatment.

[c] (2×) and [d] (1×) and corresponded to the concentration of the actinobacteria that was added to the culture medium where 1× was 7.3 x 10^6 CFU/ml.

[e] (1×) corresponded to the concentration of the *Bacillus* that was added to the culture medium where 1× = 9 x10^6 CFU/ml.

*Refers to the value which is statistically different relatively to the control (one- sample *t*-test, $P < 0.05$).

In general, the ability of a combination of compatible isolates to promote *L. minor* frond numbers was found to be equal or lower than the ability of the single strains composing the consortium (Fig 2).

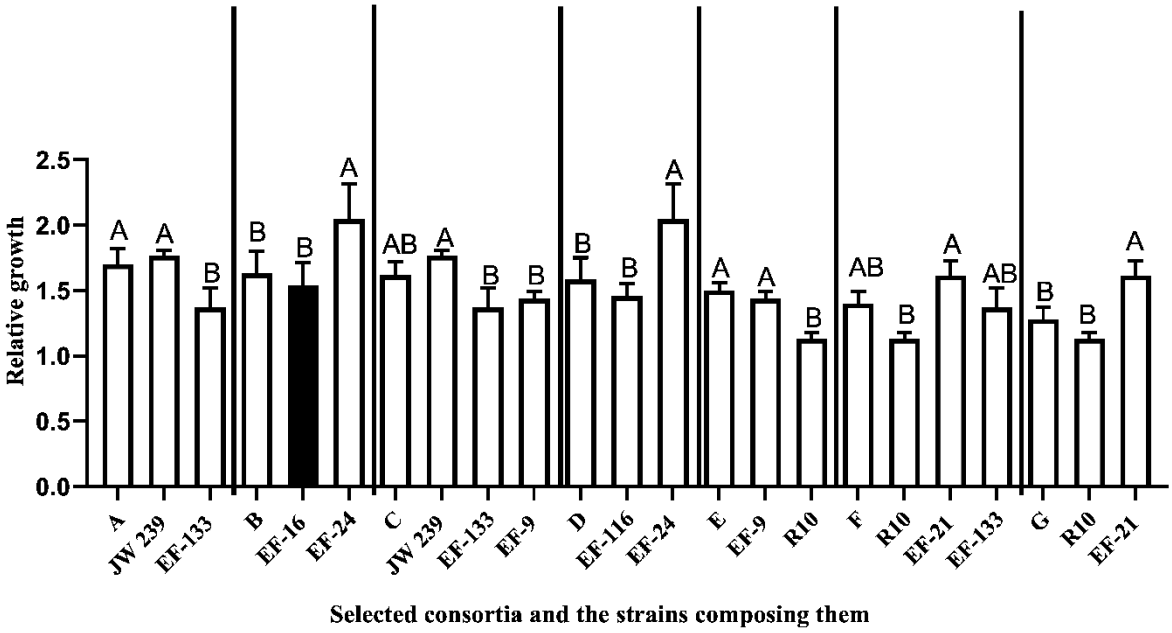

Figure 2. Comparison between the consortia and the strains composing them depending on the relative growth values of *Lemna minor*. The white bars refer to the value which is statistically different from the control and promoted *L. minor* growth (one-sample *t*-test, $P < 0.05$). Bars accompanied by the same letter are not statistically different from each other. Vertical error bars represent the standard deviation.

Growth promotion assay on lettuce

Consortia A and E were tested for their capacity to promote lettuce growth at two different concentrations (10× and 100×) but no effect on lettuce growth was found at the concentration 10× (data not shown). Consortium A promoted the growth of the lettuce seedlings and significantly increased their dry weight by 46% relatively to the control by applying it at the concentration 100×. The effect was found to be statistically equal to the effect of the actinobacterial strain JW 239 which significantly increased the dry weight by 36% (Fig. 3A). The other strain composing this consortium (EF-133) did not significantly increase the dry weight (Fig. 3A).

Consortium E also promoted the growth of lettuce seedlings (Fig. 4). It significantly increased their dry weight by 52% and its effect was found to be greater than the effect of each single strain composing it (Fig. 3 B).

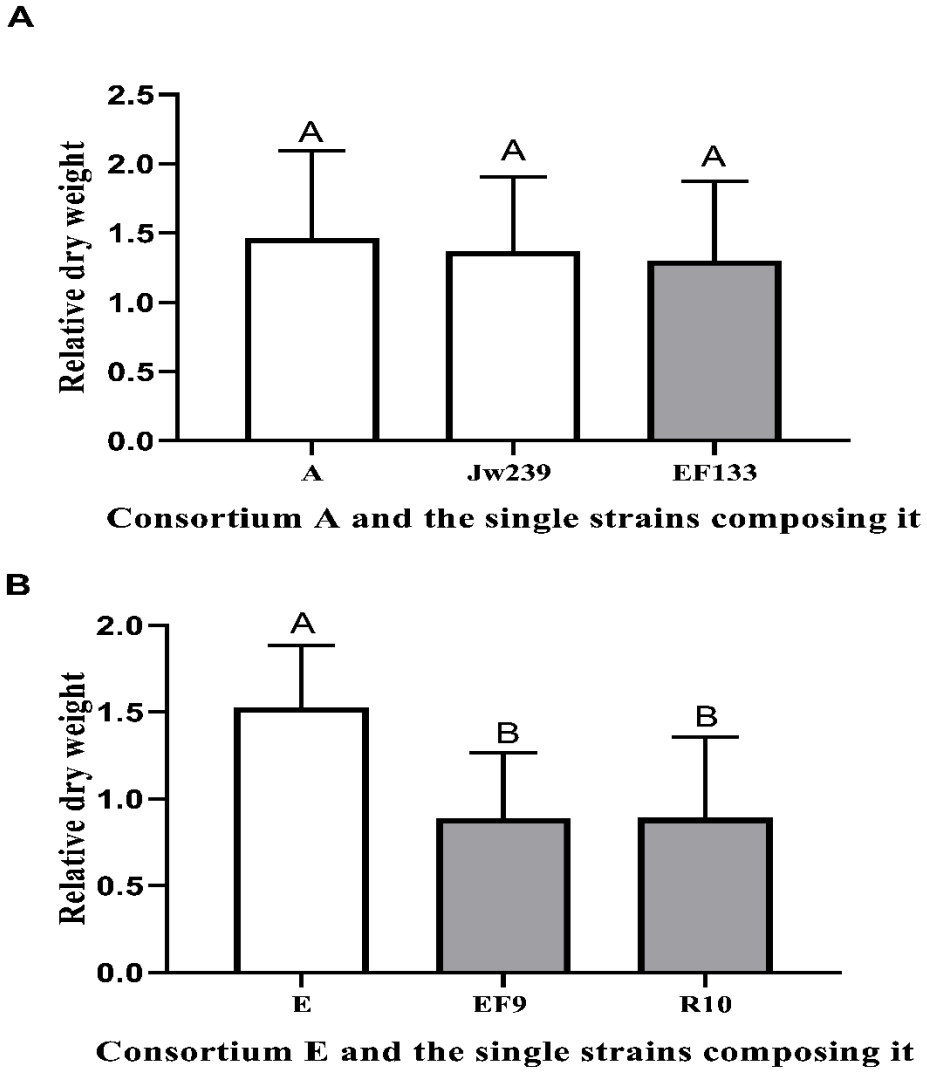

Figure 3. Effect of consortia A and E and each single strain composing these consortia on the relative growth (± S.D.) of lettuce. In (A) and (B) white bars refer to the value which is statistically different relatively to the control and promoted the growth of the lettuce seedlings (*t*-test, $p < 0.05$). Bars accompanied by the same letter are not statistically different.

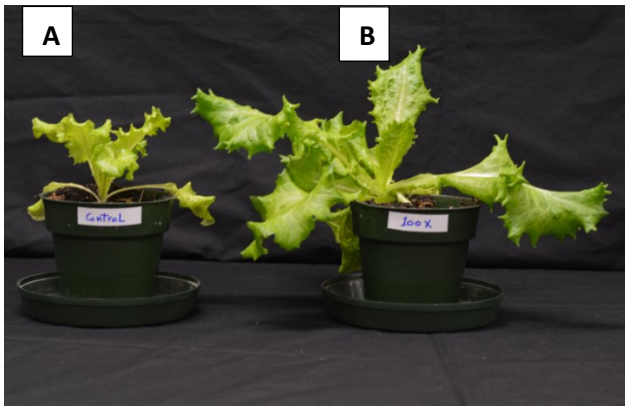

Figure 4. Uninoculated control (A) and lettuce inoculated with consortium E at concentration 100× after 55 days of growth (B). This photo is representative of 5 replicates.

Determination of the taxonomic identity of the strains composing the selected consortia

Partial sequencing of the 16S rRNA gene revealed that the actinobacterial strains composing the selected consortia belong to *Streptomyces* species (Table 4) while *Bacillus* strain R10 showed 99.5% sequence similarity with *Bacillus thuringiensis* after sequencing of the *gyrA* gene (Table 4). The sequences were deposited in the GenBank database under accession numbers from MK757244 to MK757251 (Table 4).

Table 4. Identification of the strains composing the selected consortia

Strain	GenBank accession number	Nearest GenBank neighbour	GenBank accession no. of the neighbor	Identity (%)
R10	MK757244	*Bacillus thuringiensis* strain L-7601	CP020002	99.6%
EF-16	MK757245	*Streptomyces griseoaurantiacus* strain NBRC	NR_041186	99.6%
EF-21	MK757246	*S. griseoaurantiacus* strain AC38	KY412831	99.5%
EF-116	MK757247	*S. griseoaurantiacus* strain BB9	KT274756	99.7%
EF-133	MK757248	*Streptomyces olivochromogenes* strain xsd08157	FJ481073	99.4%
JW 239	MK757249	*Streptomyces lividans* strain KUMB-A5	KY767029	99.5%
EF-9	MK757250	*Streptomyces badius* strain HLF4	MK156399	100.0%
EF-24	MK757251	*S. lividans* strain YLA0	KT362142	99.8%

Supplementary Table S1. Colorimetric screening of auxin produced by the actinobacterial and the *Bacillus* strains

Actinobacterial strain	Optical density at 530 nm
EF-38, EF-31 and NC-1498	0.11
NC-1344, ZX7 puk 6403 and EF-47	0.12
NC-2013, JW 239 and A3(2)	0.19
Vic8, Lac3, EF-107 and JJY4	0.13
ATCC 23916	0.23
MG1655	0.34
E4 mélan -	0.46
ESS2368, EF-6 and EF-24	0.16
EF-101, EF-23, ML-1, GRA-12	0.15
EF-116	0.24
ML-5, C-4, EF-129	0.18
FP-60, ML-4, CEK-018 #10	0.15
EF-138	0.27
EF-100	0.09
nev11, T9 mélan -, Ref8 and D-1	0.10
EF-17, N106, 89-01-04 #118 and GRA-13	0.23
EF-40	0.44
EF-9, LC-5 and ATCC 25435	0.12
Euro #6	0.33
ATCC 21840, EF-11 and EF-21	0.11
24 mélan -	0.29
TK-24	0.13
LE-2 #135	0.58
GRA-7, D4	0.25
GRA-9, EF-16, EF-120 and X-6	0.10
CG-1	0.37
GRA-15, C-3, EF-133 and EF-108	0.14
CG-3, EF-74, and EF-79	0.16
CG-4	0.34
EF-49	0.06
EF-39, D4, D3 and EF-136	0.11
EF-58	0.21

EURO # 3, GRA-24, X-1and EF-43	0.17
GRA-23	0.26
EF-43 and C-8	0.22
EF-32, EF-90, EF-13 and EF-54	0.14
TAS-18a #51	0.38
AC 2055 and EF-21	0.12
EF-89	0.20
GRA-12	0.28
Bacillus strain	Optical density at 530 nm
L5	0.27
L6 and L7	0.25
L8	0.14
R1	0.53
R12	0.34
R13	0.16
R10	0.33
L3	0.12

Discussion

Actinobacterial and *Bacillus* strains isolated from soil, rhizosphere and phyllosphere were screened for IAA production. The proportion of the tested *Bacillus* strains (75%) to produce IAA was found to be higher than the proportion of the tested actinobacterial strains (29%). A high proportion of auxin-producing *Bacillus* was previously recorded (Ali et al., 2009), while it was reported that two third of the proportion of tested actinobacterial strains isolated from the rhizosphere produced auxin (Harikrishnan et al., 2014; Abd-Alla et al., 2013). In contrast, only one third of the actinobacterial strains tested here produced auxin.

The selected auxin producing actinobacterial and *Bacillus* strains were tested for growth promotion on *L. minor* as it was reported that the plant growth can be promoted by auxin-producing bacterial strains (Vidal-Quist et al., 2013; Bhutani et al., 2018). The proportion of the selected actinobacterial strains (73%) to promote *L. minor* frond numbers was found to be higher than the proportion of the selected *Bacillus* strains (11%) despite the higher proportion of *Bacillus* strains to produce auxin compared to actinobacterial strains. Auxin-producing *Streptomyces* strains studied revealed PGPR capability, corroborating observations made by (Gopalakrishnan et al., 2013, Khamna et al., 2010). In *Bacillus,* it was reported that auxin production was not a good predictor of PGPR potential (Etminani and Harighi, et al., 2018; Akinrinlola et al., 2018). In our case, auxin production capability and low PGPR potentials were observed in the *Bacillus* strains studied.

Previous studies reported the growth promotion capacity of *Bacillus thuringiensis* on a variety of crop plant species (Goes, 2012, Raddadi et al., 2008) owing to its efficiency to exhibit plant growth promoting traits (Raddadi et al., 2008). This corroborate with the present study as the *B. thuringiensis* strain R10 that produced auxin, promoted *L. minor* growth.

In the present study, after trying all possible bacterial combinations, only 19% were selected depending on the compatibility between the strains and the efficiency of each strain composing each combination to promote *L. minor* growth individually. Formulating bacterial consortium is not an easy task. Molina-Romero et al., (2017) reported that only one consortium composed of four compatible PGPR strains was made from 20 strains selected after compatibility assays and their efficiency in promoting maize growth. All the strains composing the 14 selected consortia were found to promote *L.* minor growth by mono-inoculation. In contrast, not all of them promoted the growth when put together as a consortium. Seven bacterial consortia (out of the 14) promoted *L. minor* frond numbers indicating that different bacterial consortia differ in their ability to promote *L. minor* growth. Also, it was reported by Ishizawa et al., (2017) that the capacity of 15 bacterial consortia to promote *L. minor* frond numbers varied from positive to negative effects.

The capacity of a combination of compatible isolates to promote *L. minor* frond numbers was found to be equal or lower than the ability of the single strains composing the consortia. This may be due to the absence of synergy between the consortia members and their competition for nutrients (Nihorimbere et al., 2011; Radić et al., 2010). The present study also agrees with previous studies reporting that the effect of combined inoculation to promote plant growth was seldom better than the mono-inoculation. In fact, it has been reported that the effect of different bacterial consortia were found to be

similar to the effect of the individual strains on growth promotion of *L. min*or (Ishizawa et al., 2017), and that the effect of co-inoculation of two PGPR strains to promote *L. minor* growth was lower than their mono-inoculation (Yamakawa et al., 2018). Our study contradicts other previous studies that reported that the co-inoculation of PGPR strains had a higher capacity to promote tomato growth compared to their mono-inoculation (Oluwambe and Kofoworola, 2016) and also, it was reported that the inoculation of a consortium of three PGPR strains showed more growth promotion in rice compared to the single strains composing the consortium (Nandakumar et al., 2001).

Consortia A and E promoted the growth of both *L. minor* and lettuce. A high concentration of inoculum from both consortia (100×) was needed to promote lettuce growth but the lower concentration (10×) did not promote growth. Results suggest that growth promotion of lettuce depended on the inoculum concentration (Suckstorff and Berg, 2003, Bonaterra et al., 2003). Except for the *Streptomyces* strain JW 239, the individual strains composing consortia A and E did not promote lettuce growth.

Consortium E promoted the growth of lettuce seedlings and its effect was found to be greater than the effect of each strain composing it individually. This could be explained by the synergistic interactions among members of this consortium (Egamberdieva et al., 2016; Rojas et al., 2000; Armada et al., 2016) as it is possible that its members have been participating in the availability of nutrients (Shrestha et al., 2007), occupying different niches within the plant, creating a cooperative bacterial consortium (Kamilova et al., 2005) and promoting plant growth through different mechanisms (Holguin and Bashan, 1996). In conclusion, using PGPR consortia composed of actinobacterial and *Bacillus* strains has proven more efficient to promote plant growth compared to the mono-inoculation. Moreover, *L. minor* has shown to be a good model plant to evaluate

PGPR potential of consortia, as two consortia promoted both *L. minor* and lettuce growth.

Future efforts will be necessary to compare the plant growth promotion capacity of the selected bacterial consortia with that of a fertilizer. Moreover, consortia A and E presented desirable traits which might suggest promise for future field application to promote the growth of lettuce and perhaps other crops, thus contributing to sustainable agricultural practices.

Selection of strains for the establishment of efficient bacterial consortia as inoculants is a critical step (Hassen et al., 2016) that needs several co-interaction experiments with the strains composing the consortia, followed with co-inoculation trials to determine their promotion potential to promote plant growth (Sundaramoorthy et al., 2012; Singh et al., 2014; dos Santos et al., 2017). The aim of the present study was to establish PGPR consortia using actinobacteria and *Bacillus* isolates. Bacterial consortia composed of actinobacterial strains that promote plant growth are commercially available to farmers worldwide (Vurukonda et al., 2018). To our knowledge, this is the first article reporting bacterial consortia composed of actinobacterial and *Bacillus* strains promoting plant growth.

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CHAPTER 3

GENERAL DISCUSSION AND CONCLUSION

Plant growth promoting rhizobacteria (PGPR) can display one or more mechanism for promoting plant growth. In the present study we focused on promoting plant growth by indole acetic acid (IAA) produced by PGPR. The aim of the present study was to design PGPR consortia using IAA producing actinobacteria and *Bacillus* isolates. To our knowledge, this is the first report of bacterial consortia composed of actinobacterial and *Bacillus* strains that promote plant growth.

The scientific value of this work lies in exploiting the spore-forming character of *Streptomyces* and *Bacillus* spp., which could enhance the viability of cells in future, commercially formulated products. Moreover, the combination of *Bacillus* and *Streptomyces* strains could provide significant beneficial activities for the plant, greater than the activities provided by the strains alone.

L. minor plant was used in our study as a model plant as it is characterised by its rapid growth and its numbers can double in two days. It can be used as animal fodder and organic fertilizer because of its high starch content. Moreover, it can be used in low-cost wastewater treatment systems as it can remove heavy metals and other pollutants from the water. Also, it is a promising feed stock for biofuel production because of its starch content which can improve the yields of biofuel ethanol (Ishizawa *et al*., 2017). Moreover, lettuce (*Lactuca sativa* L.) was used in the present study as a target plant. In fact, research aiming to promote *L. minor* and lettuce growth is economically relevant.

Our findings highlight the PGPR capability of the auxin-producing *Streptomyces* strains. In *Bacillus,* it was found that auxin production was not a good predictor of PGPR potential. It could be that the capacity of a strain to promote plant growth is plant species-specific (Schwachtje *et al*., 2012). This highlights the need to develop inocula tailored to the targeted plant species.

In the present study*, L. minor* was shown to be a good model plant for screening PGPR potential as two consortia (A and E) promoted both *L. minor* and lettuce growth. Co-inoculating actinobacterial and *Bacillus* strains was showed superior plant growth promotion, compared to mono-inoculation. This could be explained by the synergistic actions of the consortium members which could have promoted plant growth through different mechanisms.

Our results corroborate previous findings, as it is well-known that the ability of the consortium to promote plant growth may be lower, equal, or higher than that of the individual strains composing the consortium (Ishizawa *et al*., 2017; Yamakawa *et al*., 2018; Oluwambe and Kofoworola, 2016).

The potential of actinobacteria (alone or in consortia) to promote plant growth has been previously reported under greenhouse and field conditions, and these inocula are commercially available to farmers worldwide (Pereg and McMillan, 2015; Vurukonda *et al*., 2018; Boudjeko *et al*., 2017; Gopalakrishnan *et al*., 2013; Jog *et al*., 2012). Previous studies have reported that *Bacillus* strains, alone or in consortia, can also promote plant growth under greenhouse and field conditions (Akinrinlola *et al*., 2018; Win *et al*., 2018; Lim and Kim, 2009; Verma *et al*., 2018). Single, commercially-available *Bacillus* strains are also known for their capacity to promote plant growth (Pereg and McMillan 2015; Brannen and Kenney, 1997; Ngugi *et al*., 2005; Cawoy *et al*., 2011).

Additional research will be necessary to determine which mechanisms are responsible for plant growth promotion by the selected consortia. It would be interesting to compare plant growth promotion potential of the selected bacterial consortia and a fertilizer by conducting different treatments such as: PGPR consortia alone, chemical fertilizer, PGPR and reduced chemical fertilizer, and non-treated control plants, in both greenhouse and field studies, in an effort to reduce the use of chemical fertilizers . Consortia A and E presented desirable traits which suggests their future field application could enhance the growth of lettuce and perhaps other crops. An important next step would be to determine if the selected consortia could improve the growth of crops in natural environments, as this would contribute to sustainable practices in agriculture.

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